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REVIEW

Designing stapled peptides to inhibit protein-protein interactions: An analysis of successes in a rapidly changing field

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Abstract

Two decades after their discovery, stapled peptide methodologies have evolved to a point where they can be used with confidence to generate therapeutic leads. Research groups across the world are testing innovative methodologies for their design, with dozens of publications released every month. A number of stapled peptide drug candidates have recently entered clinical trials. In this review, we provide an overview of successful methods for their construction, highlight trends in the deposited crystal structures of stapled peptide complexed to their targets and discuss properties that contribute towards improved pharmacological profiles.

KEYWORDS

analysis, crystal structure, design, stapled peptides, therapeutics

1 | INTRODUCTION

Despite heavy investment in the early 21st century, a number of challenges have stalled the development of marketable peptide therapeutic drugs. As a consequence, between 2003 and 2019 only 13 synthetic peptide drugs reached the European market.^[1] But with 140 peptides in clinical development in 2019,^[2] interest in this class of therapeutics is clearly growing and constrained peptide technologies have attracted attention from larger pharmaceutical companies as well as academic laboratories and smaller start-ups.^[3] Short peptides with natural aminoacids usually have poor drug-like properties since they frequently have high conformational variability, low cell penetration, and undergo rapid proteolysis; while longer peptides can be challenging both to produce and deliver in cells. Some of the earliest peptide drugs developed over 50 years ago, were natural hormones such as insulin,^[4] erythropoietin,^[5] oxytocin,^[6] secretin^[7] and calcitonin,^[8] which all have comparatively high molecular masses (3-7 kDa). However, shorter peptides

were also marketed during the same period; including vasopressin,^[9] a natural 10-mer peptide hormone. Notably the first seven FDA-approved peptidic drugs (Insulin, Adrenocorticotrophic hormone, Calcitonin, Oxytocin, Vasopressin, Octreotide and Leuporelin)^[10] all had a stabilised, or constrained, secondary structure, which is linked to improved resistance to proteolysis. Thus, stabilisation of secondary structure, be it through the introduction of non-peptidic fragments, backbone modifications, or unnatural amino acids to the sequence has been a prominent feature of peptide drug development since the 1990s.

Stapled peptides, whereby cross-linking of two or more side-chains is carried out via chemical synthesis, generally have a more compact structure, enhanced cell penetration, and are more resistant to proteolysis.^[11-13] Moreover, their metabolites are relatively safe,^[14] and recent studies suggest that stapled peptides offer an advantage over traditional drugs by averting the development of drug resistance.^[11,15] Hence stapled peptides have predictably sparked a growing interest from the scientific community since the early 2000s

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and have emerged as a potential new class of drugs. A 2019 review by Ali *et al.* found 78 stapled peptides where structural information has been submitted to the protein databank (PDB),^[16] of which 55 also had binding affinity reported. In parallel, the chemistry landscape for stapled peptides is continuously diversifying with a dozen “novel” linker chemistries reported in publications in 2019.^[17–23] In some instances, only very subtle differences in the staple chemistry, or stereochemistry, trigger a significant change in the binding affinity, the pharmaceutical profile of the peptide, or give added functionality.^[21,24–29] Despite multiple publications and reviews describing reliable methods for the synthesis of stapled peptides^[30–35] and the commercialisation of the most common unnatural amino acids, their synthesis remains costly and until a few years ago, non-automated. Consequently, the synthesis and screening of stapled peptides have usually been restricted to a few dozen peptides, limiting access to extensive libraries.

Stabilized peptide scaffolds sit at the interface of biologics and small-molecule drugs, and guidance for their development can be found in both fields. In this review, we focus on the successful approaches that have been published for the design of stapled peptides that inhibit specific protein-protein interactions (PPIs), and do not cover the design of stapled peptides that act as antimicrobial peptides (AMPs). Since many stapled peptides are designed to inhibit *intracellular* PPIs, they necessarily share common features with another class of peptide therapeutic, the cell-penetrating peptides (CPPs).^[15,18,36–40] We cover some of the notable strategies that have been used to optimise the pharmacokinetic properties of stapled peptides sequences, enhance their activity and reduce their proteolysis. But whilst we have focussed this review on these enhancements, noting the expanding interest of pharmaceutical companies for novel peptides,^[41] in some cases more “classical approaches” might provide a better alternative to stapling.^[42,43] Rather than highlighting solely standard PPI targets (e.g., p53-MDM2/MDMX,^[44–48] BCL9/ β -catenin,^[49] BCL-2[BAX]/BCL-X_L,^[50] HIV gp41/10E8 antibody,^[51] LEDGF-p75/HIV-IN)^[52], we have included a broad range of examples from the very recent literature to show how widely peptide stapling is being adopted.

2 | SECTION 1: DEFINING THE PRIMARY SEQUENCE

Cell signalling is achieved principally through a cascade of protein-protein interactions that assemble functionally related proteins into complexes, activating signal transduction pathways. The protein interaction network of an organism, or interactome, generally gives a better indication of its biological complexity than its genome. In 2017, Hutlin *et al.*^[53] estimated the number of candidate interactions in the human interactome as 56 000; specificity at the binding interface is thus essential to ensure cell functionality. As for natural peptides, the specificity of stapled peptides is largely ensured by the choice of the amino acids interfacing with the target protein. The synthetic landscape is vast: using the 20 natural amino acids to build peptides of up to five residues gives over 3 million possible sequences. The synthesis of stapled peptides is more challenging than linear peptides, as they often contain unnatural amino acid (uAA) residues that are not biosynthesized by bacteria, and can be costly to prepare. In this section, we have analysed recent successes in the design of stapled peptides to identify specific techniques used for their primary sequence optimisation (Figure 1).

2.1 | Natural peptide interaction sequences

Starting from a lead peptide with poor pharmacokinetics and improving its properties by using stapling techniques is the most obvious route to advance the development of peptide therapeutics. Multiple successful examples have been reported in which stapling has been achieved across a variety of secondary structures such as helical peptides, beta-hairpins, and “extended” peptides.

In 2017, Wu *et al.*^[56] investigated the cell penetrant helical peptide, melittin. Melittin is a naturally occurring peptide from honeybee venom that has been found to inhibit the proliferation of hepatocellular carcinoma (HCC) cancerous cells. Wu *et al.* prepared a library of stapled peptides by truncation and optimisation of the staple position and length, using Verdine's ring-closing metathesis method for all-hydrocarbon staple production.^[57] The stapled peptides only differed in the length of the

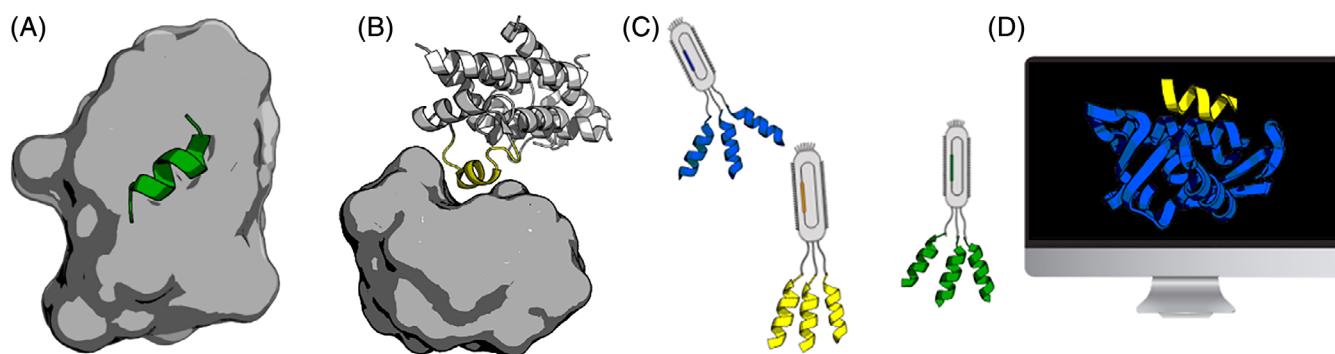


FIGURE 1 Approaches to defining the primary sequence of stapled peptides. A, Starting from a natural peptide interaction sequence. B, Excising a helical fragment from a protein-protein interface. C, Developing stapled peptide sequences using screening. D, Computational design of stapled peptide primary sequences

truncated peptide, the residues replaced by the staple, and the length of the staple linker. Some of the stapled peptides exhibit enhanced binding affinity and significantly improved resistance to proteolysis over the parent peptide. The enhanced helicity of a stapled peptide in solution often promotes its activity; stapled peptides based on the sequence of helical, estrogen receptor (ER) coactivator peptides, show a marked increase in helicity and enhanced K_d relative to the initial peptide while retaining an almost identical binding mode.^[58] The HIF-1 α /p300 PPI plays a key role in tumour metabolism. The HIF-1 α peptide is a 40-mer containing three alpha-helical segments each of which binds p300 at a specific site. By focusing on the largest helical segment, and using a dibromomaleimide stapling strategy, a competitive binder of the HIF-1 α peptide with increased helicity in the bound state was reported (Figure 2A).^[54]

Nonhelical stapled peptides have also been developed in recent papers targeting nuclear PPIs. Wiedmann *et al.*^[55] used peptides based on an HNF1 β NLS sequence developed in the 1990s by Lin *et al.*^[59] and “double click” CuACC staple chemistry,^[60,61,62] to produce a series of constrained extended stapled peptides (Figure 2B). Initial bis-triazole stapled peptides had reduced binding affinity for their importin protein target relative to the peptide lead. After further optimisation of the linker length, a stapled peptide with an equivalent low micromolar K_d was generated. Crucially, cell penetration was only observed for the stapled peptide. McGrath *et al.* have also designed a constrained peptide that binds to the WDR domain of Transducin-like Enhancer (TLE). The design was based on a peptide derived from a transcription factor binding partner.^[63] Stapling this short peptide was achieved by linking the indole moiety of the N-terminal tryptophan to the C-terminal proline via ring-closing metathesis. X-ray analysis showed that the binding mode was almost identical to that of the initial peptide with an approximately 6-fold higher affinity of the stapled peptide over its acyclic counterpart.^[59]

includes one short contiguous protein segment, this becomes a convenient starting point for the development of a therapeutic peptide. If the secondary structure of the interacting protein segment promotes the positioning of the PPI interacting residues, peptide stapling is most likely to reproduce the native contacts. Indeed, McWhinnie *et al.* have reported three hydrocarbon-stapled peptide series which reproduce the secondary structure of the native protein they were extracted from (α Syn, CKS1 and CK1 α) with high fidelity, whereas the unstapled natural sequences displayed almost no secondary structure content (Figure 3A).^[64] Similarly, Wang *et al.* have isolated a minimum helical motif from the coiled-coil region identified in the crystal structure of the respiratory syncytial virus fusion (RSV-F) protein,^[65] and then enhanced the helicity of this initial peptide sequence using computational mutagenesis and all-hydrocarbon peptide stapling, achieving a corresponding 2-fold improvement in binding affinity.

Successful examples of a peptide excision strategy applied to the generation of all-hydrocarbon stapled peptides include: stapled peptides which block the helix-helix interfaces found in the coiled-coil region of the gp41 protein with potent anti-viral activity against HIV-1;^[67] stapled peptides which mimic the junction domain of *Plasmodium falciparum* calcium-dependent protein kinase 1 (PfCDPK1; Figure 3B) to disrupt J-domain binding and provide allosteric inhibition of PfCDPK1 activity, blocking malarial parasite development;^[66] stapled peptides extracted from the dimerization interface of bone morphogenetic protein-2 (BMP-2) which is a possible target for bone repair therapies;^[68] stapled peptides which target the nuclear transcription factor NF- κ B, a therapeutic target implicated in various diseases such as cancers and neurodegeneration.^[69] Clearly, the success of these strategies has relied on the availability of crystallographic data and amino acid building blocks to readily synthesize stapled peptide sequences.

2.2 | Excision of a helical fragment from a protein-protein interface

Frequently structural data for a PPI implicated in a disease is available, but no peptide binders have been reported. If the binding interface

2.3 | Stapled peptide sequences from biological screening

Until very recently, many popular biological approaches used for the development of peptide therapeutic leads (e.g., phage display,^[70]

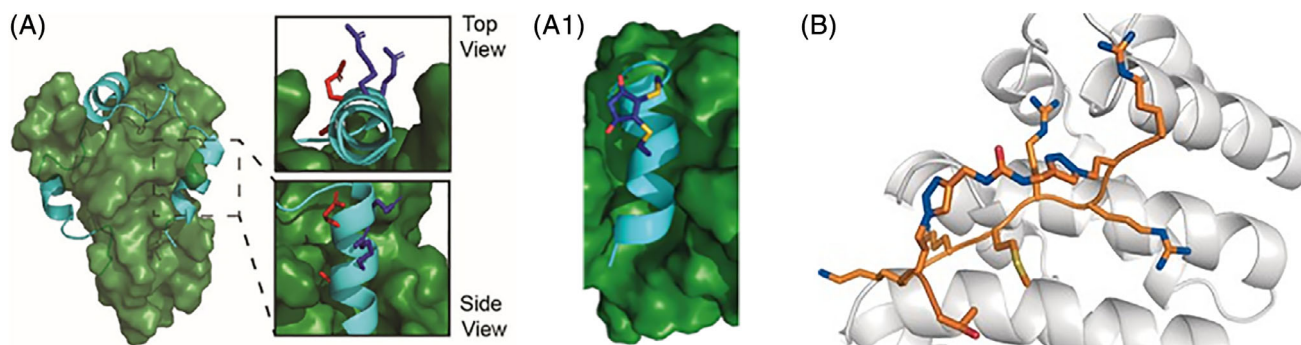


FIGURE 2 Peptide conformations constrained by stapling. A, Crystal structure of HIF-1 α (blue) binding to p300 (green); A1, Model of a stapled peptide (blue) derived from HIF-1 α binding to p300 (green) reproducing similar contacts. Reproduced from *Chem. Eur. J.* 2020, 26, 7638–7646, under Creative Commons Licence CC-BY. B, Crystal structure of an extended stapled peptide (orange) derived from the HNF1 β NLS peptide which binds to mImportin α 1. Reproduced from *Angew. Chem. Int. Ed.* 2017, 56, 524–529, under Creative Commons Licence CC-BY

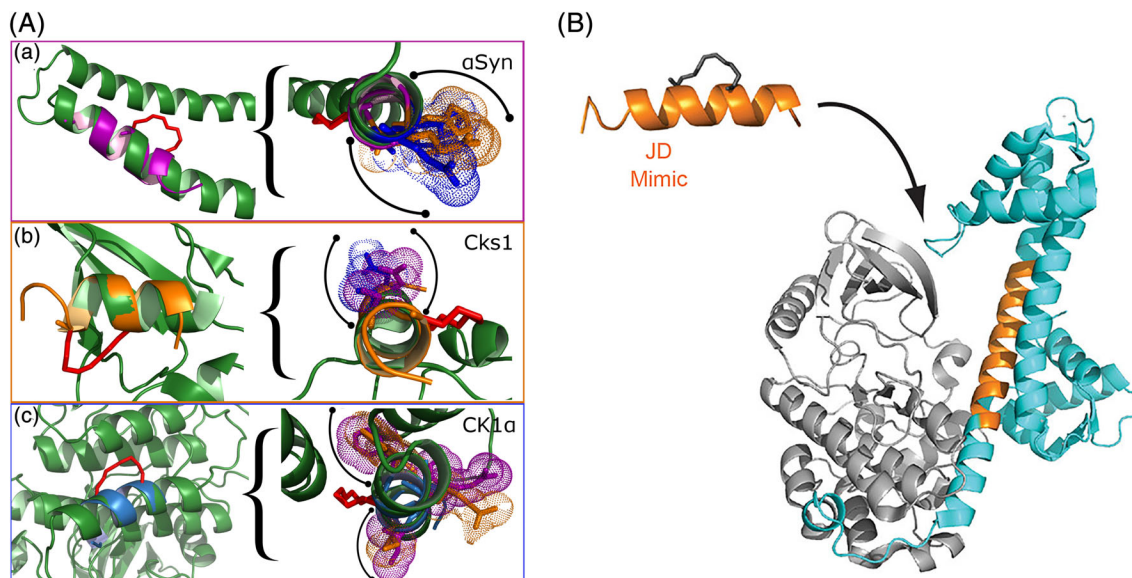


FIGURE 3 Excision strategies in the design of stapled peptides. A, NMR Structures of three stapled peptides derived from (a) α Syn (purple), (b) Cks1 (orange) and (c) CK1 α (blue) aligned with their initial protein segment (green). PDB codes 1XQ8, 2AST and 1CK1_A. Reproduced with permission from Chem. Eur. J. 2018, 24, 2094-2097, Copyright 2018 John Wiley and Sons. B, Design of a stapled peptide (orange) from the excision of the junction domain of *Plasmodium falciparum* Calcium-Dependent Protein Kinase 1 (orange) in a catalytically inactive state. Calmodulin-like domain and kinase domain shown in blue and gray respectively. PDB code 3KU2. Reproduced with permission from ACS Infect. Dis. 2019, 5, 506-514, Copyright 2019 American Chemical Society

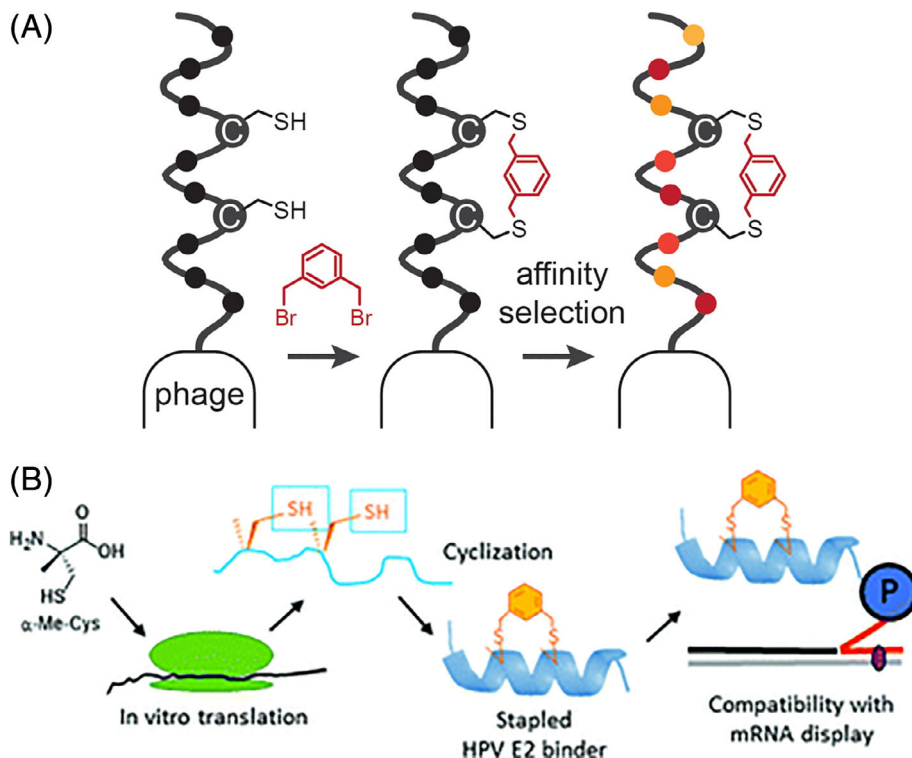
ribosome display,^[71] mRNA-display,^[72] bacterial or yeast surface display^[73] could not be applied to the development of stapled peptides due to the need to incorporate unnatural amino acids, and stapling reaction protocols harmful to bacteria or phages. Instead, such screening methodologies are used to identify initial peptide leads that are then optimised into stapled variants. To illustrate, Achek *et al.* discovered the first peptide inhibitor of TLR4, a key target for the treatment of rheumatoid arthritis, using phage display techniques and lactam stapling which increased the helicity of the initial peptide and improved its IC₅₀ 2-fold over its unstapled variant.^[74] Developments in stapling methodologies now allow biological screening to be applied to stapled peptides, where the staple is introduced through cysteine alkylation. In this instance, no uAA incorporation is required, and the cross-linking reaction can be conducted under mild, dilute conditions that are not deleterious to phages. Using a phage library based on the axin α -helix (Figure 4A), Diderich *et al.* were able to generate a cysteine-stapled peptide PPI inhibitor for β -catenin which showed nanomolar activity in a fluorescence polarization competition assay.^[75] Using the same approach with biphenyl linkers, Ananachatkul *et al.* produced micromolar binders of Galectin-3.^[76] The cysteine alkylation approach has been extended to the incorporation of α -methyl substituted cysteine using a strategy based on the mRNA display of peptides (Figure 4B). This uAA has been shown to increase the helical propensity of the cysteine-stapled peptide, giving similar properties to related all-hydrocarbon stapled peptides with α -methyl substitution.^[77] Since mRNA display can create libraries that are several orders of magnitude larger than on-bead or phage display libraries, this could be a very powerful strategy for the generation of new stapled peptide leads.

2.4 | Computational design of stapled peptide sequences

Analysis of structures of protein-peptide complexes derived from X-ray diffracted crystals, NMR measurements or molecular dynamics (MD) simulations is another effective approach to design of stapled peptide sequences (Figure 1D). The most straightforward analyses rely on interactive 3D visualisation of structures using popular molecular graphics software such as Pymol.^[78] The position and spacing of hydrogen bonds and hydrophobic contacts at a binding interface allow key residues to be inferred. When multiple structures of related peptides are available it may be possible to deduce conformational changes that occur upon binding linear sequences to direct staple optimisation.^[79,80] A complementary computational approach to this structure-based design of stapled peptides involves extracting functionally significant patterns from the sequences of naturally occurring peptides and re-using these leads to design *de novo* peptides. This type of bioinformatics approach requires large databases of peptides with known activity and consequently has not yet played a major role in stapled peptide design, owing to the comparatively small number of such datasets in the literature. However, fast and reliable methods such as AGADIR^[81] could potentially be used prior to the introduction of staples to enhance the overall propensity of a peptide sequence to fold into an α -helix.^[82] The reader is directed to an extended review of AMP databases and data mining by Porto *et al.* for further details.^[83]

MD simulations enhance structure-based visualisation methods for peptide design by providing information about molecular

FIGURE 4 Adapting biological methods to the rapid screening of stapled peptides. A, A cysteine crosslinking stapling procedure allows phage display screening of stapled peptides without the need for uAA incorporation. Reproduced with permission from ACS Chem. Biol. 2016, 11, 1422–1427, Copyright 2016 American Chemical Society. B, The display of peptides incorporating α -methyl-substituted cysteine on mRNA allows screening of large libraries of stapled peptides. Reproduced from Chem. Commun. 2019, 55, 8959–8962, under Creative Commons Licence CC-BY-NC, published by The Royal Society of Chemistry



flexibility, and estimates of binding energetics. MD simulations may be used on their own, but also in conjunction with other computational methods, such as free energy calculations, metadynamics, steered MD, parallel MD and other methods which overcome limitations inherent to Boltzmann sampling.^[84–89] In theory, any MD method for the design of peptides could be adapted to stapled peptides. However, a major limitation currently is the availability of high quality parameter sets to model the energetics of unnatural amino acids. To date, only a few MD methods have been applied to stapled peptides. Fragment-based approaches, such as those applied to the development of small molecule binders and to predicting binding pockets, have been successfully transposed to the design of stapled peptides by Tan *et al.* who used a fragment screen on MDM2 to optimise the placement of both aromatic and staple residues in their peptide lead (Figure 5A).^[90] The MELD accelerated sampling technique, which uses a Hamiltonian and temperature replica exchange approach, accurately predicts binding poses of stapled peptides where key interactions are known, and also predicts the binding energy of peptides (Figure 5B).^[91,92] Free energy perturbations (FEP) can be used to improve a given sequence; typically a virtual alanine scan is conducted and the free energy is computed to measure the effect of changes,^[93] or to enhance the binding properties of the peptide.^[94] Despite advances in FEP methods for macrocycles,^[95,96] application of this method to stapled peptides could suffer from complications if the mutations occurred at the staple residues. As an example, Valiente *et al.* describe the use of FEP methods for sequence selection, combined with umbrella sampling calculations to estimate binding free energies of the modelled stapled-peptides to the target receptor BCL_{XL}.^[97] The binding energy between a peptide and protein

can also be estimated with the Molecular Mechanics/Poisson Boltzmann Surface Area (MM/PBSA) or Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) methods.^[98] In these methods, molecular dynamics simulations of the receptor-ligand complex in explicit, or implicit, solvent are run to calculate the free energy of solvation of the complex. A few examples of the application of MM/GBSA to stapled peptides have been reported, which helped understand the role of hydration and flexibility in stapled-peptide binding.^[46,99–101]

Using docking algorithms to predict specific binding poses for large ligands and peptides is more challenging than for typical drug-like small molecules. First, the PPI binding surface is usually larger and lacking in distinct features, with more hydrophobic contacts than binding pockets for small molecules.^[94] Second, the conformational landscape of peptides is broader due to the large number of backbone and side-chain torsional degrees of freedoms that typically exceeds the sampling capabilities of most docking algorithms. Several methods have been designed to overcome the difficulties arising from docking peptides, such as allowing side-chain flexibility, or rearrangement, during docking.^[87] In practice, the more effective docking protocols for peptides are based on protein docking algorithms rather than small molecule docking algorithms. Applications of docking algorithms include publications by Ciemny *et al.* who successfully modelled the flexible regions during p53 peptide binding to the MDM2 protein receptor,^[102] using the CABS-dock method which accommodates large-scale structural rearrangements (Figure 5C);^[103] and Tiwari *et al.* who successfully modelled docking of the p53-activating peptide ATSP-7041 to albumin,^[46] using the protein-protein docking program ATTRACT.^[104]

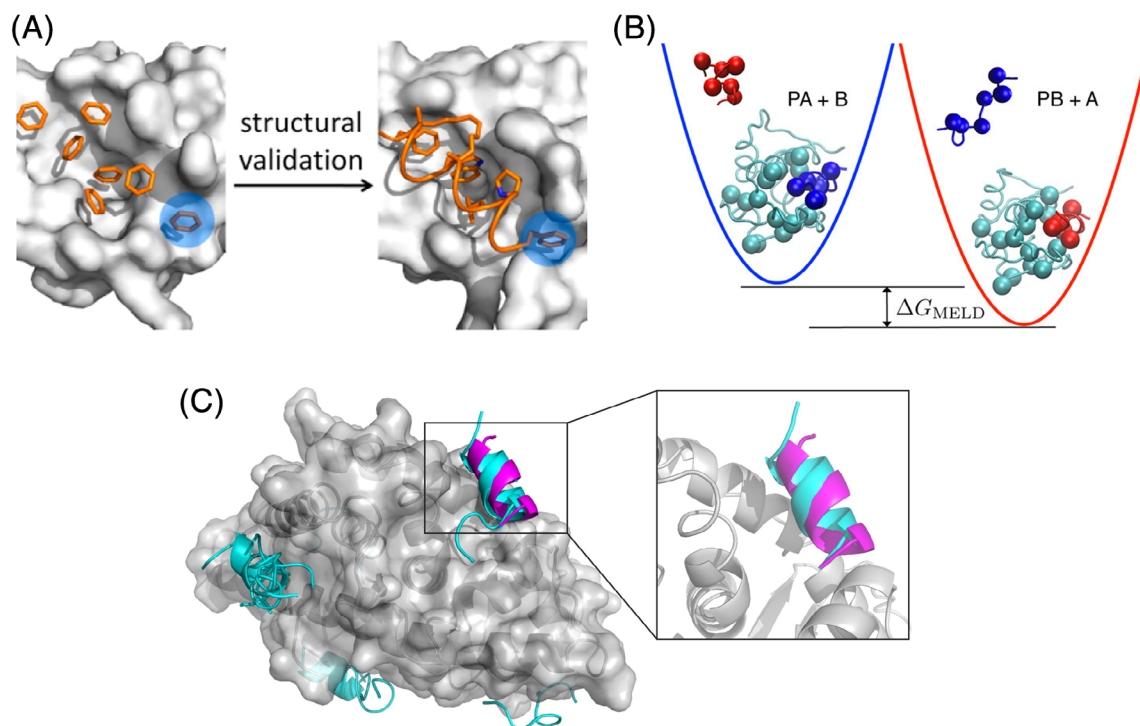


FIGURE 5 Adapting computational methods to the study of stapled peptides. A, Location of fragments observed in MD simulations (orange) shows the initial hotspots used for the design of a stapled peptide. Reproduced with permission from *J. Phys. Chem. Lett.* 2016, 7, 3452-3457, under Creative Commons Licence CC-BY, further permissions should be sought from the American Chemical Society. B, MELD sampling swaps two states: peptide A bound to the target with B in an unbound reference state (left); and peptide B bound with A in a reference state (right). The ratio of populations in the two states relates to the free energy difference ΔG_{MELD} . Reproduced with permission from *J. Chem. Theory Comput.* 2017, 13, 870-876, Copyright 2017 American Chemical Society. C, Peptide docking software, such as CABS-dock, shows that the peptide (blue) docks in the original binding site (purple). Reproduced from *Methods* 2016, 93, 72-83, under Creative Commons Licence CC-BY

2.5 | Analysis of the primary sequences of PDB-deposited stapled peptides

An analysis of crystallographic data for protein-bound stapled peptides allows several conclusions to be drawn regarding their primary sequences. The comparatively high content of unnatural amino acids reflects the relatively short length of stapled-peptide sequences (12-20 residues) that incorporate one, or sometimes two, staples (Figure 6A). Previous analysis of protein-protein interactions found that hotspots are enriched in tryptophan, arginine, and tyrosines compared to the rest of the protein.^[105] Positively charged amino acid residues are over-represented in bound stapled peptides (Figure 6B), consistent with their introduction to enhance solubility and cell-permeability.^[106-108] However, as noted by Bird *et al.*,^[109] an important balance must be struck between achieving cellular uptake by stapled peptides, while avoiding membrane disruption. Tryptophan, which seems to enhance cellular uptake through favourable membrane interactions,^[110-112] and also promotes helicity,^[113] is over-represented in stapled peptides in the PDB compared to its natural abundance in proteins. Other values are consistent with amino acids identified as either inducing (leucine)^[114] or

breaking (glycine, proline, aspartate, threonine, glutamate)^[115,116] helicity.

3 | SECTION 2: SELECTION AND PLACEMENT OF THE STAPLE RESIDUES

The selection and placement of the staple in stapled peptides requires many considerations, including how to stabilise a desired secondary structure,^[118] while retaining sufficient flexibility to allow binding; how to retain, or even enhance, biological activity through protein-staple interactions; and how to influence the activity of the stapled peptide by enhancing cell penetration. Typically these parameters are explored sequentially and as for natural peptides, libraries of stapled peptides can be constituted and scanned against the target protein via different detection methods (PCR, ELISA, NMR HSQC).^[119] For example, Araghi *et al.* optimised the sequence of MS1, a native peptide binding to Mcl-1, by first introducing an RCM staple which gave a 2-fold improvement in binding affinity.^[120,121] A library of hundreds of stapled peptides was then generated, which focussed on amino acid mutations at key positions using natural and unnatural amino

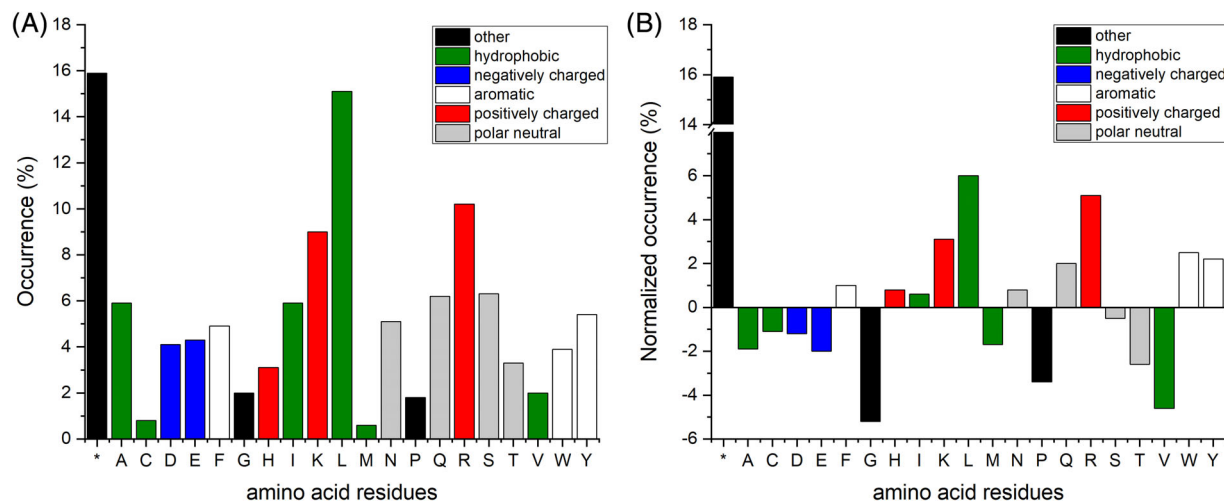


FIGURE 6 Analysis of the primary amino acid sequences of stapled peptides targeting PPIs (Supporting Information). A, Amino acid distribution in bound stapled peptides from 63 structures in the PDB (Table S2). B, Occurrence of amino acid residues in stapled peptides normalized to their natural abundance in proteins^[117]

acids; fluorescence-based on-bead screening was used for hit detection.^[120]

3.1 | Stabilising secondary structure

The alpha helix is a common motif in protein-protein recognition. A survey of the Protein Data Bank conducted in 2009, suggested that 62% of protein complexes (out of 9339) present α -helical content at their interfaces, and that less than 2% of the studied cases could be targetable using a small molecule strategy.^[122] α -Helical peptides, and helical peptides mimetics present some of the most attractive alternative approaches for the design of biological tools and therapeutics. Staple chemistries which favour α -helicity are ones which link residues spaced by one or more turns of the helix (3/4 residues for one turn, 7 residues for two turns, 11 residues for 3 turns). Due to the increasing popularity of this strategy, stapling chemistries that give rise to α -helices have been extensively reviewed in recent years.^[16,62,123–128] They can be grouped into one- and two-component strategies depending on whether the staple is formed using an external cross-linking agent or not. One-component strategies using natural amino acids consist mostly of lactam bridge formation, while those employing uAAs are dominated by all-hydrocarbon staples generated by RCM reactions between two alkene side-chains and “click” staples generated through CuAAC reactions between azide and alkyne side-chains, or thiol-ene reactions between thiol and alkene side-chains. Two-component strategies tend to rely on cross-linking two natural cysteine residues with a bifunctional linker, or on the use of double-click reactions coupling across two azide-containing uAAs. Over 75% of α -helical stapled peptides in the PDB have a clear “hydrophobic moment” with one helical face enriched in hydrophobic residues and one face enriched in hydrophilic residues (Table S2).

The introduction of a staple generally improves the therapeutic properties of individual peptides as well as their helicity, but increased helicity does not necessarily correlate with either an increase in binding affinity or in cell permeability.^[129–132] Sim *et al.* demonstrated that a staple could change the dynamics of peptide-protein interactions not only through constraining the conformation of the peptide but also by altering the hydration properties.^[133] In their computational study of the interactions of a stapled peptide with MDM2, they determined that a hydrophobic staple creates a confined space between the protein and the staple that traps waters, thus decreasing the “dewetting” barrier of the binding event. However, there are only a very few studies in which the effect of different staple chemistries on helicity have been explored (e.g., de Araujo *et al.*,^[134] Tian *et al.*)^[135], with most studies to date based on the preferred stapling methodology of the chemist generating the peptide. Thus, it is not yet possible to conclude which is the “best” staple for stabilising the structure of α -helical peptides.

While most staple chemistries have been optimised for helical peptides, some other motifs have been explored. For example, a series of stapled tankyrase inhibitors have been generated based upon a published 10 amino acid sequence; the most potent stapled peptide was shown to bind through the same key interacting residues, with a beta-hairpin secondary structure.^[101] Loops are another structural feature that can easily be achieved by multivalent linkers by thioalkylation of cysteine residues as described by Brown *et al.*^[136] and Timmerman *et al.*^[137] No specific residue spacing is required and loops of different sizes can be obtained with this method; multi-functional cysteine cross-linkers permit the formation of multiple loops. Peptides in loop conformations display a larger and more complex contact area with the protein, and usually, the linker interacts directly with the protein surface.^[101,138]

3.2 | Optimising biological activity

In general, the first step in optimising stapled peptides for a macromolecular target is the identification of appropriate sites for the incorporation of the non-natural amino acids used to form the cross-link. This is usually achieved by studying crystallographic, NMR or computational structural data; residues that are not involved in target recognition can be selected as potential sites for staple positioning, typically with $i,i + 3$; $i,i + 4$; or $i,i + 7$ spacing depending on the staple chemistry to be employed. Biological or computational alanine scanning is frequently used to determine how to minimise the effect of introducing a staple on the desired biological activity.^[43,100,121] Residues are successively mutated to alanine, and the residues conferring the least loss of activity upon mutation are chosen for the placement of staple residues. The length of linker used for the stapling process may be adjusted to match the optimum spacing identified by such scanning methods (e.g., stapling across residues spaced at $i,i + 7$ rather than $i,i + 4$).^[43,93,105,139] Lau *et al.* investigated the p53/MDM2 interaction with peptides constrained using a double click staple chemistry. Variations in the staple position and length were explored with a library of 24 peptides and a competitive fluorescence anisotropy assay to detect peptide binding.^[140] Correspondingly, Lalonde *et al.* scanned all possible lactam staple positions ($i,i + 4$ and $i,i + 7$) on a series of ghrelin peptides; testing their affinity for the growth hormone secretagogue receptor type 1a (GHS-R1a) to determine their potential use as imaging probes.^[141] Examination of deposited stapled peptide sequences in the PDB (Table S2) suggests there is no inherently preferred position for the staple in the overall amino acid sequence.

Another design consideration is matching the hydrophobicity of the staple to the protein interface. If the protein interface presents an extended, open hydrophobic surface, energetically favourable contacts with the staple may be possible. This situation is particularly favourable for all-hydrocarbon staples formed through RCM. The estrogen receptor (ER) has an extensive hydrophobic binding interface, permitting hydrophobic interactions between the all-hydrocarbon staple of stapled SRC2-BCP1 peptide with the protein (Table 1, PDB code 5WGQ).^[12] Similarly, it was shown for the non-helical stapled bicyclic peptide Grb7-B4 binding to the Grb7-SH2 sub-domain that interactions between the RCM staple and protein were required for high-affinity binding (Table 1, PDB code 5EEQ).^[138] However, staple-protein hydrophobic contacts have been observed in other types of stapled peptides, for example, to staples formed using a two-component double strain-promoted cyclisation with a cycloidyne cross-linker which also have a large hydrophobic contact surface (Table 1, PDB code 5AFG).^[142] In contrast, if the binding surface of the protein is narrower, or cleft-like, the staple may be directed towards the solvent. The binding interface of nuclear receptor coactivator 1 (NCOA1) has a more restricted surface and the hydrophobic staple of a stapled peptide derived from its binding partner YL-2 directly faces the solvent (Table 1, PDB code 5Y7W).^[143]

An analysis of crystal structures in the PDB (Table S2) indicates that staples can adopt a broad range of orientations towards the protein surface or solvent (Figure 7A). The most frequent depositions

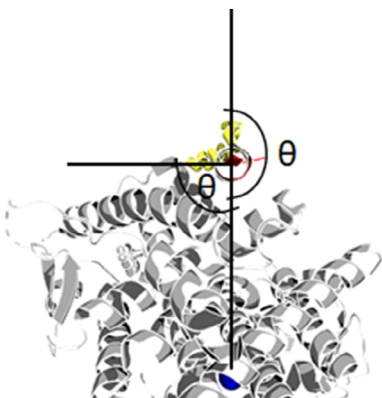
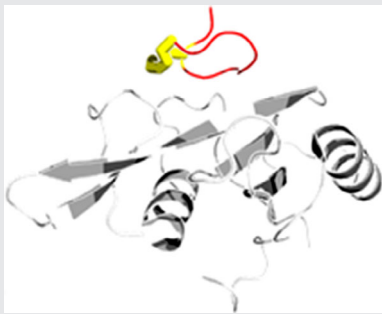
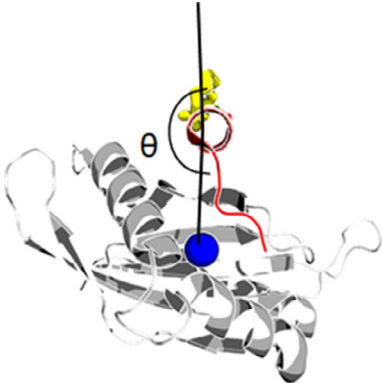
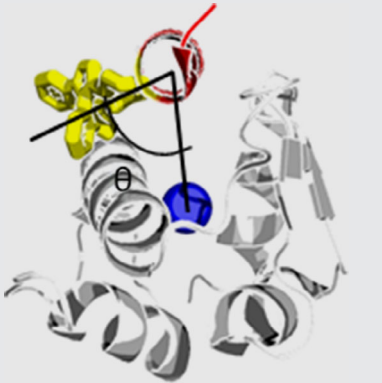
arise from the use of all hydrocarbon staples at either $i,i + 4$ or $i,i + 7$ spacing (red and blue points, Figure 7A). The surface area of the protein in contact with the stapled peptide varies from 700 Å² to 3000 Å², with an average value of 1550 Å². The mean value for the surface hydrophobicity was found to be close to zero, indicating that stapled peptides can bind both polar and hydrophobic surfaces. The staple angle (θ) is defined as the angle from the centre of mass of the peptide, to two points representing the centre of mass of the protein, and the centre of mass of the staple residues (when all three are translated into the same plane as shown in Figure 7B), and θ varies between 40° and 180°, with an average value of 103°. A weak correlation ($R^2 = 0.34$) is observed between this staple angle and the hydrophobicity of the protein surface to which it is bound. This relatively modest correlation is in part due to the difficulty in comparing bound stapled peptides, where proteins wrap around the stapled peptides differently, with one, two, or sometimes even three, interfaces with the stapled peptide. Thus, the angle of the staple residues to the protein surface is not always directly equivalent to the angle made by the centre of mass of the staple residues to the centre of mass of the protein used in this analysis. Nevertheless, there is a trend which indicates that for all staple chemistries, the more hydrophobic the PPI surface is, the more the staple will tend to lean towards it (low θ) and the more polar the surface, the more the staple will be oriented away from it (high θ). Overall this indicates that consideration of the protein surface hydrophobicity may be a useful strategy in general, but detailed investigation of specific binding site features remains important to guide staple placement.

3.3 | Ranking cell penetration by staple chemistry

In their study, Lau *et al.* developed helical peptides with nanomolar affinity to MDM2 using an $i,i + 7$ double-click stapling technique. However, some of the most potent binders of MDM2 were found to be inactive in a p53 reporter cellular assay due to poor uptake.^[142] Only a few studies have directly compared the effects of different types of staple chemistry on similar sequences, therefore it is difficult to generalise about the impact of staples on factors such as helicity, or cell uptake.^[31] Maximising helicity has been one of the principle aims of many chemical studies, but it has been shown that flexibility in macrocyclic structures is often critical in allowing a peptide to cross the cellular lipid membrane.^[145]

To compare the effect of staple chemistries, Araujo *et al.* studied the stapling of a penta-alanine peptide and concluded that a lactam bridge leads to the most helical peptides, followed by hydrocarbon and triazole staples.^[134] Tian *et al.* also concluded that for a 12-mer peptide sequence in an aqueous solution, the highest helicity was observed for lactam and all-hydrocarbon stapled peptides followed by triazole stapled peptides, while the helicity of a m-xylene bridged peptide was only increased by 10% over the natural sequence.^[135] But notably Tian *et al.* discovered that the highest cell permeability was recorded for all-hydrocarbon and perfluorobenzene stapled peptides, which also have the highest hydrophobicity; a result which was

TABLE 1 Examples of stapled-peptide protein interactions

Protein-peptide interaction	PDB Code	Staple	Staple interaction with protein surface	Staple angle θ^a	Reference
	5WGQ	RCM $i,j + 4$ and Lactam $i,j + 4$	Hydrophobic interaction of all-hydrocarbon staple with the surface; lactam staple faces the solvent	93 and 175°	12
	5EEQ	RCM $i,j + 7$	Close contacts of bis-allyl serine staple with Met495, Asp496, Asp497 backbone, and side-chains of EF loop and Ile 518 of BG loop	ND ^b	138
	5AFG	Bis click $i,j + 7$	Hydrophobic interaction of cyclodiene staple with the surface	34°	142
	5Y7W	RCM $i,j + 4$	No interaction with the protein: all-hydrocarbon staple faces the solvent	162°	143

^aStaple angle θ defined in Figure 7B.^bStaple angle θ not defined for this peptide.

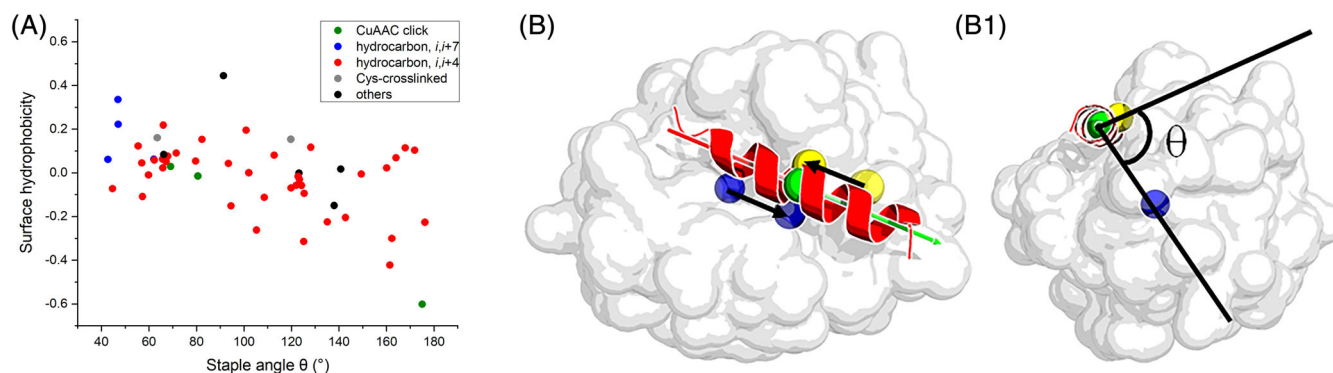


FIGURE 7 Analysis of the staple angle θ for 42 protein-bound, stapled peptide structures found in the PDB (Supporting Information). A, Plot of staple angle θ against the protein surface hydrophobicity with staple residues coloured by staple type. Surface hydrophobicity was calculated using the solvent-accessible surface area (SASA) of each amino acid present at the binding surface, multiplied by its Eisenberg value (Table S1)^[144] divided by the total area of the binding surface. More positive values denote more hydrophobic interfaces. Staple angle θ was calculated by translation (black arrows) of the centre of mass of the protein (blue sphere) and the centre of mass of the staple residues (yellow sphere) to the plane defined by the vector of the helix (red/green axis) and the centre of mass of the helix (green sphere). Illustrated for PDB structure 3MK8 in B and B1

consistent across four cell lines tested.^[135] Muppidi *et al.* used a series of aryl and vinylaryl groups to cross link $i,i+7$ cysteine residues by alkylation; they also found a correlation between cell permeability and hydrophobicity.^[146] Finally, Nielsen *et al.* found that while orally bio-available cyclic peptides frequently violate the “Rule of 5” for small molecules with a $M_w > 500$, their lipophilicity still tends to conform, with values in the range $\text{LogP} = 0$ to 5.^[147]

4 | SECTION 3: ADDING FUNCTIONALITY TO THE STAPLED PEPTIDE

Functionality may be readily added to stapled peptides through the addition of further residues to the primary sequence. For example, Dougherty *et al.* have shown that the addition of cell-penetrating peptide (CPP) sequences, can be used to enhance the cellular uptake of stapled peptides.^[148] Addition of a cyclic CPP to the C-terminus of a stapled peptide which binds MDM2, resulted in improved cytosolic delivery and cellular EC_{50} values equivalent to the small molecule inhibitor, nutlin 3a. Alternatively, the addition of E3 ligase targeting peptide sequences to stapled peptides has been explored by Jiang *et al.*, in the construction of a proteolysis-targeting chimera (PROTAC).^[149] TD-PERF, a stapled peptide with proven activity against Estrogen Receptor α (ER α), was linked to a pentapeptide which binds the Von Hippel-Lindau (VHL) E3 ligase. The resultant hetero bi-functional TD-PROTAC induced degradation of ER α in a proteasome-dependent manner inhibited the proliferation of ER α -positive breast cancer cells and led to tumour regression in an MCF-7 mouse xenograft model.

Successful strategies for functionalising the staple component of a stapled peptide require chemistry which is orthogonal to that of the peptide. It is generally easier to introduce additional functionality to staples formed using a bi-component strategy (cysteine cross-linking, double CuAAC, etc.) than their mono-component counterparts (all-

hydrocarbon RCM, mono CuAAC, etc.). Staple functionalisation may be used to add functional groups to track, or solubilise, the stapled peptide (Figure 8A); to modulate the activity of the stapled peptide (Figure 8B); or to polymerise the peptide, giving rise to multivalent species (Figure 8C).

4.1 | Attachment of functional groups

Fluorescence is often used to follow the uptake of peptides into cell and tissues and a range of methods have been developed to attach fluorescent probes to peptides.^[150] However, it has been shown that the fluorescent moiety itself can induce changes in peptide uptake.^[151] Todorovic *et al.* have developed a one-pot stapling strategy which couples a cysteine and an amine-terminated amino acid in the presence of *ortho*-phthalaldehydes forming an isoindole-bridged peptide which is inherently fluorescent (Figure 9A).^[20]

Functionalisation is not limited to fluorescence, and Wu *et al.*, describe a library of functionalised dialkynyl linkers compatible with double CuAAC stapling of peptides (Figure 9B); these include linkers with additional non-peptidic polyamines and nuclear targeting peptides.^[31] It was shown that in vitro binding affinity for MDM2 was not significantly affected by the linker chemistry; but positively charged residues enhanced both cellular uptake and target engagement in cell reporter assays.

Two different approaches to modifying the pharmacokinetics of stapled peptides through staple PEG-ylation have been explored. Xiao *et al.* used a one-component strategy to explore the effects of PEG-ylation on the conformational stability of the β -sheet WW domain, using amino acids containing both PEG motifs and an alkene function and RCM to cross-link the staple.^[152] In contrast, Tian *et al.* used a bis-cysteine alkylation strategy and a linker containing the PEG motif to probe the dual agonist activity of a series of stapled peptides against GLP-1R and GCGR (Figure 9C).^[153]

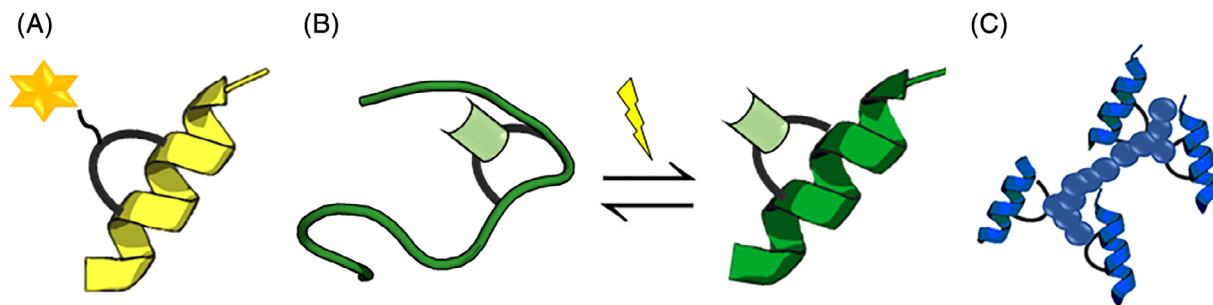


FIGURE 8 Adding functionality to the stapled peptide. A, Attachment of functional groups. B, Modulation of stapled peptide function. C, Stapled peptide polymerisation

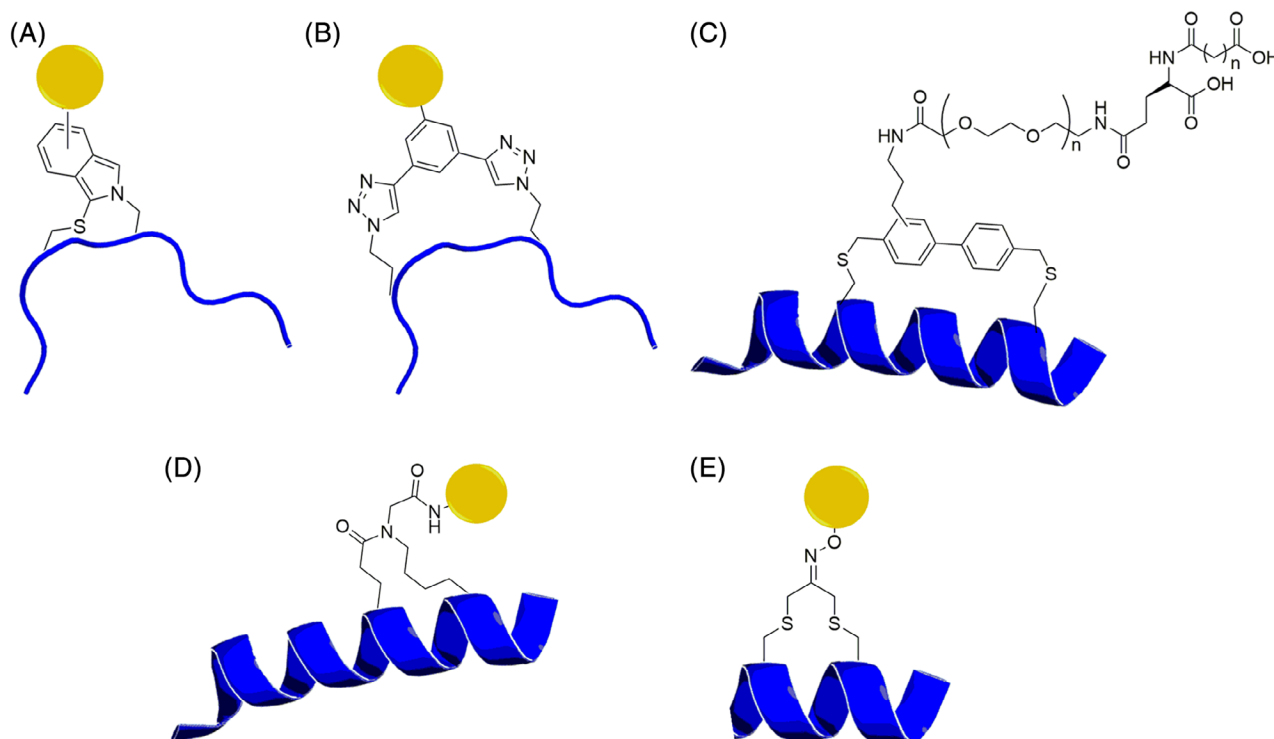


FIGURE 9 Attachment of functional groups using: A, an isoindole bridging group; B, double CuAAC stapling; C, bis-cysteine alkylation; D, an Ugi multicomponent reaction; and E, acetone crosslinking with oxime functionalisation

A large selection of chemistries have been adapted to peptides and allow some modulation for specific added functionality. Vasco *et al.* developed an Ugi reaction operating on modified side-chains can both stabilize an α -turn and introduce N-functionalization on the lactam bridge (Figure 9D).^[154] Assem *et al.* reported the chemical linking of thiol by acetone further functionalised on the ketone moiety with diverse molecular tags by oxime ligation (Figure 9E).^[155] In addition to stabilizing helical structures, this functionalisation provide opportunities to improve both peptide-target interactions and other peptide properties.

4.2 | Modulation of stapled peptide function

The helicity of stapled peptides can be made photoswitchable by incorporating a photo-isomerisable group into the staple itself. UV or visible light irradiation enables both spatial and temporal control in investigating the effects of helicity on, for example, target binding.^[156] Brendenbeck *et al.* reported the first azobenzene-based photoswitchable stapled peptide,^[157] in which the central azobenzene linker was coupled to $i, i + 7$ spaced cysteine residues via two 2-iodoacetamide groups. In a related approach, Hoppmann

et al. developed a photoswitchable azo-benzene based click amino acid which could be incorporated into peptide sequences and stapled to a cysteine residue at the $i + 4$ position using thiol-ene chemistry.^[158] In an alternative approach to photoswitching, Madden *et al.* developed a non-reversible staple which can be formed in situ by a nitrile imine-mediated cycloaddition reaction when peptides are irradiated at 302 nm.^[33] The benzyl-pyrazole group produced by this stapling process is fluorescent which allows it to be tracked in cells.^[159]

4.3 | Stapled peptide oligomerisation/polymerisation

Oligomerisation/polymerisation enhances the protease resistance of stapled peptides and provides multivalent interactions; hence several different strategies have been reported. Tran *et al.* have developed a CuAAC-based polymerisation approach in which 1,3,5-triethynylbenzene is used to staple across $i, i + 7$ spaced azido residues on the peptide. This leaves a further alkyne on the staple which can be dimerised using a bis-azido linker and a second CuAAC reaction.^[160] In an alternative approach, Lee *et al.* have developed a 'stapling polymerisation' procedure which allows the synthesis of multimers with 3 to 16 embedded helical peptides.^[161] Acryloyl groups were added to the side-chains of lysine residues at $i, i + 7$ positions in the peptide sequence and free radical polymerisation was achieved in the presence of acrylamide monomer.

5 | SECTION 4: ENHANCING THERAPEUTIC DELIVERY

The four main routes of drug administration are oral, intravenous and subcutaneous injection, and transdermal delivery (Figure 10). Oral and transdermal delivery are often considered as the best routes of administration as they are painless, non-invasive and do not require trained medical personnel.^[162] Most peptide therapeutics are subject to rapid

proteolysis, which explains their generally poor pharmacokinetics; proteases are found throughout the body, but mostly in the gastrointestinal (GI) tract, liver, kidneys, and blood.^[128] Major contributors to metabolism (e.g., trypsin and chymotrypsin) generally hydrolyse peptide bonds between specific amino acids; thus to improve short half-lives mutation and/or rearrangement of the primary amino acid sequence can be performed to avoid protease-cleavage sites. Kim *et al.* introduced single amino acid substitutions in GNU derivatives and produced AMPs resistant to proteases.^[163] Tools such as Pepcutter,^[164] which uses the initial sequence and a library of the most common proteases and their associated cleavage sites to predict the susceptibility of a peptide towards hydrolysis, can be used to optimise the primary sequence. Constraining a peptide through stapling has been shown to enhance the stability of peptides towards proteolysis in several studies.^[165,166] A second challenge for hydrophilic peptide therapeutics is rapid renal clearance from the blood, which prevents efficient uptake in tissues. Binding to albumin and other proteins reduces renal filtration, and peptide conjugation to PEG, or a lipophilic moiety, can also improve serum half-life. Pessi *et al.* produced a doubly lactam-bridged stapled peptide conjugated to cholesterol which acts as fusion inhibitor for the Ebola virus;^[167] they reported an extended serum half-life for the cholesterol-conjugated peptide allowing daily dosing. Finally, the amino acid sequence of a peptide affects its propensity to aggregate, to form either amorphous or amyloid-like fibrils; a number of programmes are now available to predict this,^[168,169] based on contributing factors such as hydrophobicity, charge state, and β -sheet forming propensity.^[168,170,171]

5.1 | Oral delivery

Oral delivery is the most frequently employed drug delivery mode, but it is also the most challenging for peptides (Figure 10A). Under the acidic conditions of the GI tract (as low as pH 1.0-3.0) some amino acids form by-products, for example cysteine, methionine and tryptophan are all susceptible to oxidation. Substitution of these sensitive amino acids (e.g., norleucine for methionine, and serine for cysteine)

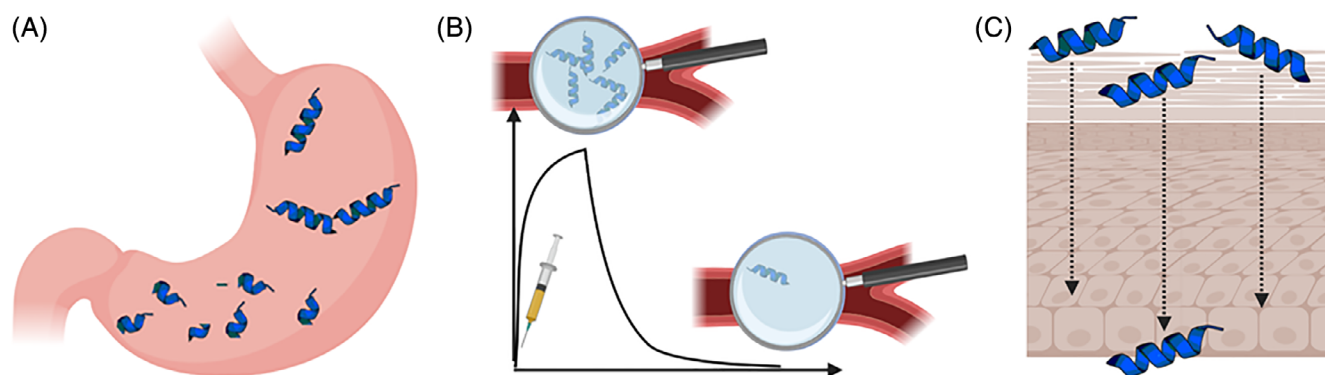


FIGURE 10 Barriers to the therapeutic delivery of stapled peptides. A, Peptides administered orally are subject to low pH and degradation by proteases; B, Peptides administered intravenously appear as a high initial burst, but are subject to degradation by serum proteases. C, Peptides administered transdermally must pass multiple layers of the epithelium

can increase peptide stability while retaining activity. N-terminal glutamine is also unstable at low pH and replacing this amino acid, or using an acetate capping group, can prevent its cyclisation.^[172,173] Orally available peptide drugs have on average 8.7 HBA and 4.5 HBD;^[174] it is thought that the highly acidic conditions of the GI tract disrupt backbone hydrogen bonding, destabilizing helical and beta-sheet conformations and making peptides more susceptible to proteases. The number of rotatable bonds in a peptide is also inversely correlated with oral bioavailability and most successful orally available peptides marketed to date are cyclic peptides (e.g., cyclosporine).^[175,176] In the case of stapled peptides, the improved stability arising from side-chain cross-linking can enable absorption, as seen with a 36-residue double-stapled peptide following oral gavage.^[177] Finally, enhanced lipophilicity can be used to increase the half-lives of peptides; fatty acid conjugation of GLP-1 analogues has been shown to increase their half-life from 10 to 12 hours to days.^[142]

As a second challenge, the gut epithelium is composed of multiple layers of cells and mucus, which the peptide therapeutic must cross. Four modes of transport are available: passive diffusion through the cells (transcellular) and through intercellular junctions (paracellular); by endocytosis followed by endosomal release; or by receptor-mediated mechanisms.^[178] Peptides with $M_w > 700$ Da have only limited paracellular permeation in intestinal epithelia,^[179,180] and an upper limit to permeation has been suggested as $M_w \sim 3.5$ kDa by Rubas *et al.*^[181,182] However, Ji *et al.* have reported the oral absorption of a 5.3 kDa cyclotide targeting intracellular MDM2.^[183] The doses used in this study were high (40 mg/kg), and the peptide was administered in 5% dextrose, which is known to enhance intestinal permeability; but evidence of activity after oral administration against this intracellular target was reported. The M_w of current oral peptide therapeutics is typically around 800 Da and does not generally exceed 2000 Da.^[174]

5.2 | Intravenous and subcutaneous injection

Intravenous or subcutaneous injections currently represent the main delivery method for peptide therapeutics.^[184] Drugs injected intravenously have a high initial bioavailability (Figure 10B) as this is defined as the proportion of a drug that is available in the bloodstream. Subcutaneous injection does lead to a slightly greater variability in bioavailability, since peptides with a higher molecular weight have been linked to a greater absorption by the lymphatic system rather than the blood.^[185,186] Overall, the most notable limitation of these delivery methods is the initial “burst” encountered when the peptide is injected, which is followed by the rapid degradation of peptide.

5.3 | Transdermal delivery

Transdermal applications include topical (skin), buccal (mouth), corneal, and intranasal delivery; as for oral administration, one of the

major challenges is transport of the stapled peptide through multiple layers of cells (Figure 10C). Enhanced cell penetration often increases trans epithelial bioavailability.^[180,187] Some transdermal drugs such as cortisone, or anti-inflammatory drugs are limited to a local distribution usually for skin or tissue-related conditions. However there are many cases where application of a drug to the skin have been proven efficient for systemic delivery (nicotine, oestrogen). Despite typically low absorption, dermal administration avoids the first-pass liver metabolism that occurs for oral drugs. The stratum corneum, constituting the top layer of the skin limits the transdermal absorption of polar and large peptides.^[188,189] The lipid structure of the stratum corneum can be disrupted by natural peptides (e.g., magainin) when these are applied in combination with a surfactant chemical enhancer leading to enhanced skin permeation.^[189] Several hundred peptides that enhance the skin permeability of drugs are collated in the database TopicalPdb.^[190] Among the reported sequences, arginine and lysine combined with lipophilic natural and unnatural amino acids are found extensively. Chemical modifications of peptides, such as the addition of lipophilic moieties, have also been shown to increase stability and skin permeability.^[167,191,192]

Transmucosal nasal drug delivery has also emerged as an important field in drug delivery technology due to the high vascularity and large surface area, which enable drug uptake.^[180] Intranasal delivery can allow the absorption of peptides of up to 2 kDa without absorption enhancers, and peptides of 2–6 kDa with absorption enhancers.^[193] Peptides can also be used as modulators to facilitate the uptake of drugs; in general, cell penetrant peptides display excellent absorption.^[194] Peptide drugs on the market using intranasal delivery include desmopressin, a cyclic peptide used to treat diabetes insipidus. However, intranasal delivery is not appropriate for the treatment of infants, for whom alternative delivery methods, such as buccal delivery, must be sought.^[195] Although stapled peptides have not yet entered clinical trials using intranasal delivery, several stapled peptides have shown efficacy in mouse models. Gaillard *et al.* reported a double-stapled RSV peptide, which disrupts viral cell entry and significantly decreases pulmonary infection in mice following intranasal delivery.^[43]

6 | CONCLUSIONS

This review has focused on the many considerations required for the design of biologically active stapled peptides, drawing from the extensive recent literature, and from analysis of PDB deposited structures containing protein-bound stapled peptides. The growing recognition of the utility of stapled peptides is fuelling the development of bespoke computational tools, synthesis procedures and biological assays, that are accelerating their adoption as a research tool. With dozens of new publications every month from research groups across the world, and several stapled peptides entering clinical trials, there is ample evidence that stapled peptide technologies will play an increasingly important role in pharmaceutical applications.

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CONFLICT OF INTEREST

Julien Michel is a current member of the Scientific Advisory Board of Cresset.

DATA AVAILABILITY STATEMENT

The data that support the analyses shown in Figures 6 and 7 are openly available from the Protein Data Bank at <http://www.rcsb.org/>.

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